Independent sources of condition dependency and multiple pathways determine a composite trait: lessons from carotenoid-based plumage colouration

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Abstract: Many colour ornaments are composite traits consisting of at least four components, which themselves may be more complex, determined by independent evolutionary pathways, and potentially being under different environmental control. To date, little evidence exists that several different components of colour elaboration are condition dependent and no direct evidence exists that different ornamental components are affected by different sources of variation. For example, in carotenoid-based plumage colouration, one of the best-known condition-dependent ornaments, colour elaboration stems from both condition-dependent pigment concentration and structural components. Some environmental flexibility of these components has been suggested, but specifically which and how they are affected remains unknown. Here, we tested whether multiple colour components may be condition dependent, by using a comprehensive 3 9 2 experimental design, in which we carotenoid supplemented and immune challenged great tit nestlings (Parus major) and quantified effects on different components of colouration. Plumage colouration was affected by an interaction between carotenoid availability and immune challenge. Path analyses showed that carotenoid supplementation increased plumage saturation via feather carotenoid concentration and via mechanisms unrelated to carotenoid deposition, while immune challenge affected feather length, but not carotenoid concentration. Thus, independent condition-dependent pathways, affected by different sources of variation, determine colour elaboration. This provides opportunities for the evolution of multiple signals within components of ornamental traits. This finding indicates that the selective forces shaping the evolution of different components of a composite trait and the trait's signal content may be more complex than believed so far, and that holistic approaches are required for drawing comprehensive evolutionary conclusions.

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INDEPENDENT SOURCES OF CONDITION DEPENDENCY AND MULTIPLE PATHWAYS DETERMINE A COMPOSITE TRAIT: LESSONS FROM CAROTENOID-BASED PLUMAGE COLORATION.

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Abstract

Many color ornaments are composite traits consisting of at least four components, which themselves may be more complex, determined by independent evolutionary pathways, and potentially being under different environmental control. To date, little evidence exists that several different components of color elaboration are condition-dependent and no direct evidence exists that different ornamental components are affected by different sources of variation. For example, in carotenoid-based plumage coloration, one of the best-known condition-dependent ornaments, color elaboration stems from both condition-dependent pigment concentration and structural components. Some environmental flexibility of these components has been suggested, but specifically which and how they are affected remains unknown. Here we tested whether multiple color components may be condition-dependent, by using a comprehensive 3 x 2 experimental design, in which we carotenoid supplemented and immune challenged great tit nestlings (*Parus major*) and quantified effects on different components of coloration. Plumage coloration was affected by an interaction between carotenoid availability and immune challenge. Path analyses showed that carotenoid supplementation increased plumage saturation via feather carotenoid concentration and via mechanisms unrelated to carotenoid-deposition, while immune challenge affected feather length, but not carotenoid concentration. Thus, independent condition-dependent pathways, affected by different sources of variation, determine color elaboration. This provides opportunities for the evolution of multiple signals within components of ornamental traits. This finding indicates that the selective forces shaping the evolution of different components of a composite trait and the trait's signal content may be more complex than believed so far, and that holistic approaches are required for drawing comprehensive evolutionary conclusions.

Keywords: Carotenoid-based ornaments, feather structure, immune challenge, trade-off, trait components, independent pathways, sexual selection, *Parus major*.

Introduction

The idea that color ornaments are composite traits determined by different evolutionary pathways has become increasingly relevant (Badyaev *et al.*, 2001; Badyaev, 2004; Grether *et al.*, 2004; Jacot *et al.*, 2010; Svensson & Wong, 2011). In particular, it has been proposed that carotenoid-based coloration, one of the best-known, condition-dependent ornaments, is determined by at least four distinct components: pigment elaboration, patch area, pigment symmetry, and patch area symmetry (Badyaev *et al.*, 2001; Badyaev, 2004). While research has mainly focused on these classic four components, few studies have investigated whether those components may be more complex, and whether independent condition-dependent pathways may determine their expression. For example, pigment elaboration, originally defined as "type and quantity of carotenoid pigments deposited in growing feathers" and measured as color hue (i.e. pigment hue, Badyaev *et al.*, 2001), includes independent effects of pigments and feather background structure (Shawkey & Hill, 2005; Jacot *et al.*, 2010). Here, we therefore use a more general terminology, that corresponds to this measure of pigment elaboration (i.e. color hue), namely color elaboration, which does not make any assumptions about how coloration is determined (note that color elaboration strictly refers to the color *per se*, excluding the extent or symmetry of the coloration, and is independent of the quantification method). Likewise, patch area ("the area of plumage (i.e., number of feathers) with carotenoid pigmentation", Badyaev *et al.*, 2001) may depend on individual feather characteristics (e.g. feather width, feather length, feather shape), number of feathers and feather arrangement (Quesada & Senar, 2006). It has been observed that different aspects of fitness could be associated with individual components of color elaboration (Badyaev *et al.*, 2001). However, evidence that these components may be determined by independent condition-dependent pathways is

indirect (Jacot *et al.*, 2010; Matrkova & Remes, 2012). Thus it is unknown whether different components may be under independent selection, which could potentially explain why carotenoid-based coloration preserves an important amount of phenotypic variability (Tolle & Wagner, 2011).

Carotenoid pigments are the main sources of the red, orange and yellow colorations present in many taxa, including fish, amphibians, reptiles, mammals, birds, crustaceans, and insects. The degree of carotenoid deposition is an important determinant of carotenoid-based color elaboration (Hill, 1992; Hill *et al.*, 1994; Hill, 2000; Hill *et al.*, 2002; Saks *et al.*, 2003; Shawkey & Hill, 2005) But robust, experimental evidence for the condition-dependency of structural aspects of ornamental feathers (i.e. feather characteristics) is to our knowledge absent. Moreover, only a handful of studies have simultaneously investigated the contributions of alternative color components (see Svensson & Wong, 2011), and very few evidence exists that different components reflect different, independent sources of condition-dependency. Therefore, it is unknown whether and how different components and their combination affect color elaboration. This, in turn, is the key to understanding how selection drives the evolution of color displays.

In birds, carotenoid-based coloration has been shown to honestly signal health/immune status (Blount *et al.*, 2003; Faivre *et al.*, 2003; McGraw & Ardia, 2003), foraging ability (Hill, 1992; Hill *et al.*, 2002), parasite load (Møller *et al.*, 2000) and nutritional condition (Hill & Montgomerie, 1994; Hill, 2000) and it may provide advantages in intra- and intersexual selection (i.e. mating success, Hill, 1999) and interspecific interactions (i.e. protection from predation, Slagsvold & Lifjeld, 1985; Delhey *et al.*, 2010). Carotenoids may also be used as antioxidants or immunostimulants, potentially leading to a trade-off between ornamental display and health (von Schantz *et al.*, 1999;

105 Lozano, 2001; Blount *et al.*, 2003; McGraw & Ardia, 2003; Alonso-Alvarez *et al.*,
106 2004; but see Navara & Hill, 2003; Fitze *et al.*, 2007; Isaksson *et al.*, 2007). According
107 to theory (Møller *et al.*, 2000; Blount *et al.*, 2003; Faivre *et al.*, 2003; McGraw, 2006)
108 carotenoid availability is limited in nature (Slagsvold & Lifjeld, 1985; Hill, 1991; 1992;
109 Andersson, 1994). Since animals cannot synthesize them de novo, the production of
110 carotenoid-based ornamentation is costly (Tschirren *et al.*, 2003b) and so honesty
111 assured (Zahavi, 1975).
112 Most research on the signaling properties of carotenoid-based plumage coloration has
113 focused on how condition-dependent color variation is caused by differences in
114 carotenoid concentration. In contrast, our experiment investigated whether different
115 condition-dependent components exist (among components of carotenoid-based color
116 elaboration), and whether they may reflect alternative and independent evolutionary
117 pathways. Using path analyses we assessed the relative importance of different
118 condition-dependent pathways and the relationships among different components.
119 Unlike previous studies, we considered a wide range of potential color determinants,
120 including structural aspects of feather design and carotenoid concentration, and
121 investigated how carotenoid supplementation and immune challenge affected these
122 components, the different measures of feather coloration, and thereby color elaboration.
123 The effect of pigment concentration on carotenoid-based coloration has been broadly
124 studied (see above), but the role of structural contributions to plumage color elaboration
125 is incomplete. Among structural features, it has been demonstrated that feather overlap
126 modifies coloration (Quesada & Senar, 2006). Evidence that color elaboration is
127 affected by condition-dependent variation in feather overlap, under natural conditions, is
128 lacking. Similarly, two studies have shown that structural aspects may be condition-
129 dependent, but it is unclear exactly which components are those and which their

130 determinants are (Jacot *et al.*, 2010; Matrkova & Remes, 2012). Moreover, the
131 activation of the immune system has been shown to alter color expression, through a
132 mechanism different from the proposed trade-off in carotenoid allocation between
133 immune function and coloration (Fitze *et al.*, 2007). The activation of the immune
134 system generally negatively affects body, wing, and wing and tail feather growth rate
135 (Klasing *et al.*, 1987; Fair *et al.*, 1999; Saino *et al.*, 2002; Pap *et al.*, 2011), but it
136 remains unknown whether it affects ornamental plumage feathers and whether such
137 differences may affect color elaboration.
138 Here, we experimentally investigated which color components are condition-dependent
139 and whether they are determined by independent pathways, using a 3 x 2 factorial
140 design where we carotenoid supplemented and immune challenged great tit nestlings
141 during early development. By using the proposed trade-off in carotenoid allocation
142 between coloration and immune function (von Schantz *et al.*, 1999) we tested which
143 color components induced changes in nestling plumage coloration. To pin down where
144 the color changes originated from, we used standardized photographic measurements
145 taken on alive birds, spectrophotometric measurements taken on individual feathers, and
146 measured several feather characteristics. We measured feather carotenoid concentration
147 using high performance liquid chromatography (HPLC) and assessed structural feather
148 components, namely feather length, feather opacity, feather development and barb
149 density. Thereafter, we used multivariate path analyses to evaluate the relative
150 contributions of these components to experimentally induced changes in carotenoid-
151 based coloration and examined whether different environmental sources and condition-
152 dependent pathways independently account for color variation.
153 If carotenoid-based coloration (i.e. color elaboration) of great tits is a complex
154 composite trait consisting of different, independent condition-dependent components,

155 we predicted that changes in plumage coloration would be the result of independent
156 contributions of carotenoid concentration and structural features of feathers, affected by
157 different environmental sources of variation.
158

Methods

Species description

The great tit is a widespread small hole-nesting passerine that breeds in woodlands and gardens across all Europe. Males and females show yellow ventral feathers, a black breast stripe, black head and neck, prominent white cheeks and olive-green upperparts. Yellow breast coloration develops early in life (Fitze *et al.*, 2003b) and depends on a combination of carotenoid pigments (lutein and zeaxanthin), mainly obtained through caterpillar ingestion (Slagsvold & Lifjeld, 1985; Partalli *et al.*, 1987), and structural components (Jacot *et al.*, 2010). Feathers develop in a sheath whose tip brakes open when the uppermost barbs are keratinized and their morphological development completed (Stettenheim, 2000). Lutein and zeaxanthin pigments are incorporated into the feather without metabolic transformation (Lucas & Stettenheim, 1972; Partalli *et al.*, 1987). As in many other avian species (McGraw, 2006), only the distal end of the feather is colored.

Experimental design

The experiment was carried out in 2001 in a great tit population breeding in nest-boxes in the Forst (46º54'N, 7º17'E/46º57'N, 7º21'E), a mixed deciduous forest near Bern, Switzerland. The experimental design and further methodological details are described elsewhere (Fitze *et al.*, 2007). To assess whether one or multiple pathways affect carotenoid-based plumage coloration, we carried out an intra-nest experiment on nestling great tits testing for a trade-off in carotenoid allocation between coloration and immune function. Nestlings were randomly assigned to two crossed treatments, namely

carotenoid supplementation and immune challenge, using a two-factorial design with three and two factor levels, respectively.

Carotenoid supplementation

The carotenoid treatment comprised three treatment groups, consisting each of two randomly chosen nestlings per nest. A first group, the βLZ group (β-carotene, lutein, zeaxanthin), was fed 2.6 mg (±0.25 mg) β-carotene beadlets (containing 8% β-carotene) and 17 mg (±0.25 mg) lutein/zeaxanthin beadlets per feeding (containing 5.58% lutein and 0.44% zeaxanthin; Hoffmann–La Roche, Basel, Switzerland), which represents the carotenoids occurring in the natural diet of great tits (Partalli *et al.*, 1987). A second group, the LZ (lutein, zeaxanthin) group, was fed 2.6 mg carotenoid free beadlets and 17 mg (±0.25 mg) lutein/zeaxanthin beadlets per feeding, which represents the carotenoids present in great tit feathers (Partalli *et al.*, 1987). Finally, the control group was fed with 19.6 mg (±0.25 mg) beadlets per feeding containing no carotenoids. Nestlings were fed every second day, starting 4 days posthatching, and thus several days before the first breast feathers appeared (P.S. Fitze personal observation). Feeding treatment ended 14 days posthatching. The supplemented amount of carotenoids and the carotenoid ratios were within the naturally ingested range (Tschirren *et al.*, 2003a).

Immunization treatment

Four days after hatching, one randomly chosen nestling of each carotenoid supplementation group was immune challenged with an intramuscular injection of 50 mL human diphtheria-tetanus (DT) vaccine (Kinder, Merieux) and 50 mL 5% rabbit red blood cells in phosphate-buffered saline (PBS), hereafter referred to as immunized group (I). The other nestling was injected 100 mL PBS and served as a control for the immunization, hereafter referred to as control-injected group (CI).

205 **Feather measurements**

206 ## Color measurements

207 In the field, we photographed nestling breast plumage coloration fifteen days
208 posthatching under standardized conditions using a digital camera (Fitze & Richner,
209 2002). Photos allowed quantifying the coloration of the plumage (i.e. color elaboration)
210 integrating coloration originating from pigment concentration and structural
211 components (i.e. feather shape, layers and arrangement). Photos were taken with
212 standardized light exposure, photographic angle, and object-objective distance. Mean
213 RGB (red, green, blue) values were obtained of 10 square measurement areas, each
214 consisting of 400 pixels, using Adobe Photoshop™. Thereafter, we calculated HSB
215 (hue, saturation, and brightness) values following the algorithm described by Foley and
216 van Dam (1984). For more detailed information on the applied methodology see
217 Fitze and Richner (2002).
218 After taking a photograph we collected 20 yellow breast feathers from the upper left
219 breast of each nestling. Feathers were kept in hermetic plastic bags and stored in the
220 dark until measurement. In the laboratory, we measured feather reflectance using an
221 Ocean Optics USB4000 spectrophotometer (range 200–850 nm; Dunedin, FL, USA)
222 with a light source (DT-MINI-2-GS) that provided light in the UV and visible range.
223 We used a reflection probe (QR400-7-UV/VIS) fixed on a reflection probe holder
224 (RPH-1) that excluded ambient light and allowed to measure reflectance at an angle of
225 90°. Reflectance was measured with respect to a white (WS-1, Ocean Optics) and a
226 black standard (black photographic cloth with no light reflectance across all
227 wavelengths). We placed five feathers on top of each other and used as a background
228 the same black photographic cloth. For each nestling, we took five measurements of the

feather tip on the dorsal side of the feather in an area of approximately 1mm^2 and for each measurement we alternated the order of the feathers in the pile (Jacot *et al.*, 2010). We computed the average reflectance of the five measurements and thereafter derived five indices describing the feather's coloration following Jacot *et al.* (2010). We calculated (1) 'background reflectance' corresponding to the absolute reflectance between 575-700nm ($R_{575\text{-}700nm}$) and being a carotenoid-independent proxy of the feather's white background structure (Jacot *et al.*, 2010), (2) 'absolute carotenoid chroma', a background structure-independent measure of carotenoid concentration ($R_{400\text{-}515nm}$/ $R_{575\text{-}700nm}$) (Jacot *et al.*, 2010), (3) UV-reflectance ($R_{300\text{-}400nm}$,)(Bennett & Cuthill, 1994), the total amount of light reflected in the UV, (4) '$R_{UVpeak'}$, the wavelength of peak reflectance in the UV (Bleiweiss, 2005), and (5) 'UV chroma', corresponding to the proportion of light reflected in the UV while controlling for differences in background structure (i.e. $R_{300\text{-}400nm}$/ $R_{575\text{-}700nm}$ (Jacot *et al.*, 2010)(Fig. 1). Photospectrometric measurements quantified feather coloration and thus color variation arising from pigments and structural feather components, and they were independent of feather density.

Structural measurements

To understand whether and how feather design affects feather coloration we measured four structural components, namely feather length, barb density, feather opacity, and developmental stage of all feathers used for the spectrophotometric analyses.

Feather length

Total feather length was measured manually with a ruler (± 0.5 mm) and corresponds to the straightened shaft length. We also measured the length of the different feather parts

252 along the feather shaft (Fig. S1 of the supporting information), including the length of
253 the yellow, white, and black colored parts and of the calamus.

254 ***Barb density***

255 For each of the measured feathers, we determined the barb density by counting the
256 number of barbs in the uppermost 5 mm of the yellow tip of the feather. This area
257 includes the spot where the spectrophotometric measurements were taken.

258 ***Feather opacity***

259 All feathers used for the spectrophotometric measurements were individually
260 photographed under standardized conditions using the same photographic setup as for
261 nestlings (Fitze & Richner, 2002). In brief, feathers were put on black photographic
262 cloth within a small box and pressed against a UV-photographic filter lens. This box
263 was placed in a standard position inside a larger opaque camera box and photos with
264 standardized light exposure and size were taken. Photos were imported into ImageJ
265 (Rasband, 1997) and two different measures of feather opacity were obtained, 1) one-
266 barb surface coverage and 2) the opacity of a feather area (Fig. S2). One-barb surface
267 coverage measures the contribution of a single barb to feather opacity, while opacity of
268 the feather area, hereafter referred to as 'feather opacity', corresponds to the surface
269 proportion covered by the barbs and barbules of the measured feather area. Prior to the
270 analysis, all photos were transformed into 8-bit black and white photos. To measure
271 area opacity we selected an area of 30 x 30 pixels within the yellow distal feather part.
272 For all feathers, the center of the square coincided with the point where the uppermost
273 barb branched off from the rachis and the sides of the sampled square were aligned
274 parallel to the shaft. We then used a grey threshold (for all feathers the same threshold)
275 to determine the percentage of the 900 pixels covered by the feather barbs and barbules.

For determining one-barb surface coverage we selected an area of 30 x 30 pixels in the middle of the barb (between the shaft and the barb tip) where no other barbs overlapped. The square was parallel aligned with the ramus and it completely fell within the barb's contour line. Surface coverage was measured using the method applied for feather opacity.

Developmental stage

Since feather development may affect opacity and feather length, and thereby feather coloration, we assigned 'development' scores to each feather used for the spectrometric measurements. Feather development was measured using a discrete scale consisting of 5 levels ranging from 0 to 4 (i. e. 0 = undeveloped feather, 4 = completely developed). Developmental scores were independently attributed to the left and right side of the feather, according to the criteria shown in Fig. S3 of the electronic supplementary information. Average developmental score was used for further analyses.

Carotenoid concentration

Feather carotenoid concentration was analyzed by HPLC using a protocol adapted from Olmedilla *et al.* (1997). In brief, 0.5-1.0 mg of feather tips were placed in 1.0 ml ethanol. The internal standard (retinyl acetate) was added and the mix was flushed with nitrogen and kept from light at 4ºC for 25 minutes. Then, the solution was placed in an ultrasound bath, with intermittent vortex, for 5 minutes. Double extraction was performed by adding 1 ml of distilled water and 2 ml of methylene chloride/hexane (1:5). Both organic phases were pooled and evaporated to dryness. The carotenoid residue was dissolved in tetrahydrofuran/ethanol (1/1) and thereafter injected into the HPLC.

The chromatographic system consisted of a Spheri-5-ODS column (Applied Biosystems, San Jose, CA) with gradient elution of acetonitrile/ methanol (85/15) for 5 minutes to acetonitrile/ methylene chloride/methanol (70/20/10) for 20 minutes. Ammonium acetate (0.025 M) was added to the methanol. Detection was carried out by a photodiode array (Model 2996; Waters Associates, Milford, MA) set at 450 nm. This method allows to simultaneously detect trans-lutein, zeaxanthin, 13/15-cis-lutein, α-carotene, all-trans-β-carotene, 9-cis-β-carotene, 13/15-cis-β-carotene, and several other carotenoids (Olmedilla *et al.*, 1997). Identification of compounds was carried out by comparing retention times with those of authentic standards and on-line UV–visible spectra. Only lutein (average ± SE: 91.66 ± 8.68 µg/g feather) and zeaxanthin (12.76 ± 6.98 µg/g feather) were detected and feather carotenoid concentration (quantity of lutein and zeaxanthin/feather mass) was used in the analyses.

Statistical analyses

Treatment effects on the different components affecting plumage coloration were analyzed with mixed-model ANOVAs using JMP® statistical package (SAS Institute Inc.) and R (R Development Core Team, 2005). Carotenoid supplementation, immunization treatment, and their interaction were modeled as fixed factors and nest was included as random factor. Residuals were tested for normality and homoscedasticity. If necessary, variables were transformed using logarithmic or arcsine squareroot transformations. Differences between treatment groups were analyzed using post-hoc LSMeans contrasts.

Using path analyses we investigated the relative contribution of three exogenous experimental parameters (carotenoid supplementation, immunization, and their interaction) on plumage coloration. All measured components were standardized and included in the path diagram. They were classified into five hierarchical levels, arranged

from the left (lowest level) to the right (highest level, Fig. 2), where each hierarchical level was determined by hierarchically lower levels and thus by those presented on their left (Quinn & Keough, 2002). For total feather length and the length of the yellow, white, or black colored feather parts the hierarchy was not clear and thus we also modeled the backward effect. Similarly, for components of the same hierarchical levels it was not clear whether and in which direction they affected each other and thus we allowed for effects in both directions. The resulting diagram (Fig. 2) shows all effects supported in ≥75% of all path models, including intermediate models resulting from backward elimination.

The path diagram was based on ten randomly chosen nests (n = 58 individuals). This was because HPLC analyses and structural feather measurements were based on this subset. Analyses on plumage coloration and feather coloration were conducted using both the subset and the full sample size of 295 individuals, from 54 nests. For comparisons between individuals belonging (1) or not (0) to the subset, we modeled subset as a factor. There were no significant differences in body size and body condition between subsets and no significant interactions between the applied treatments and subsets (all $P > 0.5$).

Results

Interaction between carotenoid supplementation and immunization treatment

There was a significant interaction between carotenoid supplementation and immunization on plumage saturation, plumage brightness (Table 1, Fig. 3), and one barb surface coverage ($F_{2, 43} = 3.75$, $P = 0.032$, 7.6% of variance explained). There was also a significant interaction in plumage saturation ($F_{2, 216} = 3.85$ $P = 0.022$) in the full data set. The effect of carotenoid supplementation depended on the immunization treatment. Immunized individuals of the βLZ group showed significantly reduced plumage saturation ($F_{1, 43} = 28.67$, $P < 0.001$) and plumage brightness ($F_{1, 43} = 5.98$, $P = 0.019$) but no differences existed between immunization groups in the LZ and C groups ($P > 0.1$). There was a significant negative effect of immunization in the C group on one barb surface coverage ($F_{1, 43} = 6.99$, $P = 0.011$), but no significant differences between I and CI nestlings existed in the βLZ and LZ group ($P > 0.1$). There were no significant interactions in any of the other color parameters, in feather carotenoid concentration, and in structural components (all $P \geq 0.1$).

Carotenoid supplementation

Effects on plumage coloration

There was a significant effect of carotenoid supplementation on plumage hue and saturation, but not plumage brightness (Table 1, Fig.3). Plumage hue was lower in the LZ compared to the βLZ (LSMeans contrast: $F_{1, 46} = 4.96$; $P = 0.031$) and the C group (LSMeans contrast: $F_{1, 46} = 15.21$; $P < 0.001$) and tended to be lower in the βLZ compared to the C group (LSMeans contrast: $F_{1, 46} = 2.99$; $P = 0.080$). Thus, LZ and

potentially also βLZ nestlings produced plumages with more orange tones. Plumage saturation of the carotenoid supplemented groups was significantly higher than in the C group in both immunization groups ($F_{1,43} \geq 28.67$; $P < 0.001$) and it was significantly higher in the LZ group compared to the βLZ group, in the CI group ($F_{1,43} = 4.42$; $P = 0.041$; also see Table 1). Similar results were found when using the entire data set (effects of carotenoid supplementation on hue: $F_{2,216} = 38.89$; $P < 0.001$; saturation: $F_{2,216} = 102.23$; $P < 0.001$; brightness: $F_{2,216} = 2.09$; $P = 0.126$; Table S1).

Effects on feather coloration

Carotenoid supplementation significantly affected 'absolute carotenoid chroma' in both data sets (Table 1, $F_{2,215} = 18.44$; $P < 0.001$). Both carotenoid supplementation groups (βLZ and LZ) showed reduced 'absolute carotenoid chroma' compared to the C group (LSMeans contrasts: all $F_{1,46} \geq 22.64$; all $P < 0.001$), indicating increased light absorption between 400-515nm and thus more incorporated carotenoids (Jacot *et al.*, 2010). Carotenoid supplementation did not affect 'background reflectance' (Table 1, $F_{2,215} = 0.96$; $P = 0.382$). In the subset, carotenoid supplementation significantly affected 'UVchroma', but not UV-reflectance and 'R_{UVpeak}' (Table 1). However, when using the full dataset, carotenoid supplementation significantly affected all three variables (all $F_{2,215} \geq 5.38$; $P \leq 0.005$, $\geq 2.4\%$ variance explained, Table S1), as predicted by a previous study (Jacot *et al.*, 2010). This indicates that detecting carotenoid effects on UV properties requires large sample sizes because carotenoid reflectivity is relatively small in the UV wavelength. Group C showed significantly more UV-reflectance, higher 'UVchroma', and higher 'R_{UVpeak}' than the LZ and the βLZ group (all LSMeans contrasts: $P < 0.05$). There were no significant differences between the LZ and the βLZ group (all LSMeans contrasts: $P \geq 0.1$).

Effects on feather carotenoid concentration

Carotenoid supplementation significantly affected feather carotenoid concentration ($F_{2,43}$ = 7.79; P = 0.001, 16.5% of variance explained). Significantly higher concentration was observed in the carotenoid supplementation groups (LSMeans contrasts: βLZ vs. C: $F_{1,43}$ = 13.31, $P \leq 0.001$, estimate βLZ: 18.945 µg/g ± 7.466 SE; LZ vs. C: $F_{1,43}$ = 8.65, P = 0.005, estimate LZ: 10.478 µg/g ± 7.584 SE) and there were no significant differences between the βLZ and LZ group (LSMeans contrast: $F_{1,43}$ = 0.44; P = 0.512).

Effects on feather design

None of the structural feather components was significantly influenced by the carotenoid treatment (all $P \geq 0.1$).

Immunization treatment

Immune challenged nestlings had significantly shorter breast feathers than control nestlings (6.8% of variance explained, Fig. 4) and tended to show reduced feather opacity ($F_{1,43}$ = 3.65, P = 0.061, estimate -1.030 ± 0.554). There were no significant effects of immunization on any of the other components (feather and plumage coloration in the subset and the complete data set, carotenoid concentration and other components of feather design, all $P \geq 0.1$).

Path analysis

Path analyses revealed that carotenoid supplementation affected plumage coloration through carotenoid concentration but also independently of it (Fig. 2). Increased carotenoid supplementation positively affected plumage saturation and feather carotenoid concentration, and negatively affected 'absolute carotenoid chroma', the latter being due to reduced light reflection in the 400-515nm wavelength. Additionally, it affected plumage saturation through its effect on carotenoid concentration, which

414 negatively affected ‘absolute carotenoid chroma’. In line with the results of Jacot *et al.*
415 (2010), the effect of carotenoid supplementation on ‘UV chroma’ observed in the mixed
416 ANOVAs (Table 1), was via ‘absolute carotenoid chroma’. Immunization negatively
417 affected total feather length and feather opacity. There was a significant positive
418 correlation between total feather length, and the length of the differently colored feather
419 parts, which in turn affected UV-reflectance and plumage hue, in the case of yellow
420 length and both yellow and white lengths, respectively. Reduced feather UV-reflectance
421 affected ‘UV chroma’ and thereby plumage saturation. The interaction between
422 carotenoid supplementation and immunization affected plumage brightness. Immune
423 challenged individuals of the βLZ group ($F_{1,43}$ = 11.93, P = 0.001, estimate -0.014 ±
424 0.005) showed reduced plumage brightness, but not individuals of the other groups.
425 There was also an interactive effect on feather ‘UV chroma’, which was reduced in the
426 immune challenged βLZ group ($F_{1,30}$ = 7.55, P = 0.010, estimate -0.119 ± 0.042) but not
427 in the other groups, and on ‘absolute carotenoid chroma’, which was higher in the
428 immune challenged βLZ group than in all other groups ($F_{1,30}$ = 5.61, P = 0.024, estimate
429 0.137 ± 0.056). ‘UV chroma’ positively affected saturation and ‘absolute carotenoid
430 chroma’, which affected plumage brightness and saturation.
431

Discussion

It has been proposed that carotenoid-based ornaments are determined by at least four distinct components: pigment elaboration, patch area, pigment symmetry, and patch area symmetry (Badyaev *et al.*, 2001; Badyaev, 2004). Recent studies suggest that these components may themselves be more complex than originally described (Jacot *et al.*, 2010; Matrkova & Remes, 2012) but condition-dependency of carotenoid-based ornaments have mainly focused on how coloration reflects variation in one component (e.g. in carotenoid concentration; Saks *et al.*, 2003; McGraw & Gregory, 2004; Senar *et al.*, 2008). To date there exists few and no direct evidence that different condition-dependent components may be affected by different, independent evolutionary pathways.

We carried out an experiment using great tits, testing whether different components of color elaboration are determined by multiple and/or independent pathways of condition-dependency and whether they mirror different sources of condition-dependency. We analyzed treatment effects on the different components affecting coloration and determined their relative contribution to intraspecific variance in color elaboration using path analyses. Immune challenge reduced plumage saturation in the βLZ group (Fig. 3), , which is in line with the proposed trade-off in carotenoid allocation, and suggests that nestlings with an activated immune system (those of the βLZ group, see Fitze *et al.*, 2007) used less carotenoids for coloration. However, only pigment availability (carotenoid supplementation), but not immune challenge or their interaction, affected feather carotenoid concentration and 'absolute carotenoid chroma' (Table 1). Therefore, the interactive effect on color elaboration (saturation) was carotenoid-concentration independent. This confirms that the proposed trade-off between coloration and immune function for rare carotenoids does not account for reduced plumage saturation in

457 nestlings with an activated immune system (see Fitze *et al.*, 2007). Reduced plumage
458 saturation could however be explained by shared pathways between trait production and
459 vital cellular processes that determine condition (Hill, 2011). This shows that plumage
460 coloration was the consequence of complex feather carotenoid concentration dependent
461 and carotenoid-independent effects and thus that alternative pathways exist. Carotenoid
462 supplementation led to higher feather carotenoid concentration, which decreased
463 'absolute carotenoid chroma' of the feathers, and thereby increased plumage saturation
464 (Fig. 2). Moreover, independent of feather carotenoid concentration, carotenoid
465 supplementation decreased 'absolute carotenoid chroma' and increased plumage
466 saturation (Fig. 2; see arrows directly connecting treatment with measures of plumage
467 coloration). This suggests that the effects of carotenoid supplementation (Table 1) are
468 the result of alternative pathways. The interaction negatively affected plumage
469 brightness (i.e. the βLZ, I group showed reduced brightness; Fig. 2) independently of
470 the measured color components, reduced 'UV chroma', which affected saturation, and
471 positively affected 'absolute carotenoid chroma', which in turn increased plumage
472 brightness and decreased plumage saturation. Therefore, the interaction had, at the same
473 time, negative and positive effects on color intensity (brightness), suggesting that its
474 overall effect (Table 1) may depend on the strength of each pathway and thus it may not
475 always lead to reduced color intensity.
476 Given that the interaction affected 'absolute carotenoid chroma' and 'UV chroma', its
477 effects on feather coloration were the consequence of chromatic color changes,
478 suggesting that the chromatic part of feather coloration is not exclusively carotenoid
479 determined. This argument is supported by the fact that the immunized βLZ group
480 exhibited higher 'absolute carotenoid chroma' than the control injected βLZ group (Fig.
481 3). If solely carotenoid incorporation modified the chromatic part of the coloration,

differences between the I and CI groups of the βLZ treatment would be the result of carotenoids and thus we would have also expected higher 'UV chroma' in the I compared to the CI group of the βLZ (Jacot *et al.*, 2010; Fig.1; see methods). In contrast, a negative interactive effect was observed on 'UV chroma' (Fig. 2). The fact that carotenoid supplementation and its interaction with immunization had carotenoid concentration independent effects on plumage coloration, suggests that these experimental effects may have also been the result of other, here undetermined components affecting coloration, such as subjacent variation in skin coloration (Jourdie *et al.*, 2004). Immunization reduced total feather length, which was, in turn, positively correlated with the length of the white and yellow feather parts, and thereby affected hue and saturation (Fig. 2). Differences in feather length could lead to different number of overlapping breast feathers, which has been shown to affect chromatic and achromatic coloration (Quesada & Senar, 2006). This indicates that immunization altered plumage coloration through an independent pathway, different from the pathways by which carotenoid supplementation or the interaction affected plumage coloration. Additional support for this alternative pathway stems from the lack of effect of carotenoid supplementation (and the interaction) on feather length and any other component of feather design.

The effect of the immune challenge on feather length (Fig. 4) is congruent with the effects observed on wing and tail length (Brommer *et al.*, 2011; Maenniste & Horak, 2011; Pap *et al.*, 2011) and showed that structural features of color elaboration are under environmental control and thus that they are also condition-dependent traits, which contribute to environmentally-induced carotenoid-based plumage color variation (Fitze *et al.*, 2003a). Interestingly, although immunization affected feather length, no effect of immunization on overall plumage coloration was found in the mixed model ANOVAs

that do not take into account the hierarchy of the color determinants shown in Fig. 2. This suggests that immunization effects on plumage coloration caused by reduced feather length may have been cancelled out due to opposing effects of other components of plumage coloration and thus that complex interactive effects may exist, that are not necessarily consistent across environments (Sillanpää *et al.*, 2010). None of the treatments affected 'background reflectance' even though it has been shown to be partly determined by environment-related factors (Jacot *et al.*, 2010; Matrkova & Remes, 2012). Thus the ultimate determinants of feather background structure remain unknown. The results stress the important, but not exclusive, contribution of carotenoids to condition-dependent color elaboration. Although carotenoid supplementation and immunization affected plumage coloration with similar strength (i.e. had similar β's - arrow width-, Fig. 2), the former affected plumage coloration through several mechanisms, while immunization affected plumage coloration through feather length. Thus, condition-dependent color elaboration is clearly not the sole result of differential carotenoid incorporation into the feathers, suggesting that environmental determination of this trait (e.g., Slagsvold & Lifjeld, 1985; Eeva *et al.*, 1998; Hõrak *et al.*, 2000; Møller *et al.*, 2000; Fitze *et al.*, 2003b; Tschirren *et al.*, 2003a; b) is the result of simultaneous and alternative (pigmentary or not) pathways and complex interactions. This shows that, at least in carotenoid-based coloration, color elaboration is a more complex composite trait, affected by several different condition-dependent components and their associated alternative pathways, with independent sources of variation. This result also suggests that color elaboration of other types of coloration, may be the result of independent pathways under differential control. For example, in melanin-based coloration, patch area may be the result of differential melanization and/or feather length. Our results may also have important implications for fish, reptiles and

amphibians, where the basic chromatophore unit is composed of three different layers consisting of different types of pigments, which may bear different information and may have evolved independently (Grether *et al.*, 2004). Evidence stems e.g. from the common lizard (*Lacerta vivipara*), whose carotenoid-based ventral coloration shows condition-dependent chromatic color changes which are independent of carotenoid intake, suggesting that their carotenoid-based coloration may as well be a composite trait determined by different pathways (Fitze *et al.*, 2009; San-Jose *et al.*, 2012; San-Jose *et al.*, in press). Carotenoid-based colorations observed in nature are ubiquitous and include, besides feather coloration, beak and leg coloration of birds, hair and skin coloration in other animals, and petal, leaf, stem and fruit coloration in plants. Thus, in both the animal kingdom and in plants, condition-dependent ornaments, whose expression is influenced by several factors, may indeed be the result of multiple condition-dependent components, determined by different condition-dependent pathways.

Our results support a new perspective on the evolution of color traits, where selection may be acting to maintain a balance between the different components affecting the display (Grether *et al.*, 2005; Svensson & Wong, 2011). On one hand, different selective pressures may affect different components of an ornamental trait, and those may thus evolve independently (e.g. feather length and carotenoid incorporation). On the other hand, different selective pressures may similarly affect different components, potentially leading to the incorrect conclusion that one selective pressure may be at the origin of their evolution. For example, in several species sexual selection has been suggested to favor individuals with yellower plumages since yellowness provides information about health status, body condition, or parental quality (Dufva & Allander, 1995; McGraw & Ardia, 2003), and at the same time, selection for longer feathers

provides an advantage in natural selection due to improved insulation/thermoregulation, which may indirectly lead to increased feather overlap, and thus, yellowness.

In conclusion, we show that inferring evolutionary explanations using only one component can lead to simplistic or inaccurate conclusions. Using an integrative approach, we provided the first experimental evidence for condition-dependency of both, pigmentary and structural features of plumage coloration of great tits, and the existence of different, independent pathways shaping color elaboration of carotenoid-based coloration, revealing a previously ignored level of complexity of color composite traits. The condition-dependent nature of several of their components may favor the evolution of multiple-component signals. The existence of different condition-dependent mechanisms that independently affected color elaboration may provide an explanation why different fitness optima may exist, why selection may not deplete genetic variance, and it may explain inconsistency among studies.

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Table 1

Table 1: Effects of the mixed model ANOVA on plumage coloration (A) and feather coloration (B) of the subset sample (n = 58). The test statistics are given. When significance was found, only results of the backward eliminated final model are shown and the percentage of variance explained (%) is given. The initial full model included two fixed factors: carotenoid supplementation and immunization, and their interaction.

A. PLUMAGE COLORATION

Color parameter	Treatment	*F*	d.f.	*P*	%
Hue	Carotenoid supplementation	7.67	2, 46	0.001	17.2
Saturation	Carotenoid supplementation	28.70	2, 43	<0.001	36.0
	Immunization	0.01	1, 43	0.930	
	Interaction	4.54	2, 43	0.016	5.7
Brightness	Carotenoid supplementation	2.21	2, 43	0.122	5.9
	Immunization	0.36	1, 43	0.549	
	Interaction	4.17	2, 43	0.022	10.9

B. FEATHER COLORATION

Color parameter	Treatment	*F*	d.f.	*P*	%
Absolute Carotenoid chroma	Carotenoid supplementation	12.65	2, 46	<0.001	18.7
UV-reflectance	Carotenoid supplementation	0.69	2, 43	0.351	
	Immunization	0.32	1, 43	0.574	
	Interaction	1.07	1. 43	0.351	
UV chroma	Carotenoid supplementation	3.64	2, 46	0.034	1.6
R_{UVpeak}	Carotenoid supplementation	0.08	2, 43	0.919	

	Immunization	0.21	1, 43	0.649
	Interaction	0.41	2, 43	0.668
Background	Carotenoid supplementation	0.12	2, 43	0.885
reflectance	Immunization	0.77	1, 43	0.384
	Interaction	1.15	2, 43	0.327

586

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743

Figure Legends

Fig. 1: Reflectance spectra of breast feathers of great tit nestlings. Shown are measurement ranges of five indices describing feather coloration. Measures of total reflectance include (a) UV-reflectance measured between 300-400nm, and (b) 'background reflectance' measured between 575-700nm. Reflectance, independent of variance in the background structure, include 'UV chroma' (a/b) and 'absolute carotenoid chroma' (c/b), whereas λ at peak reflectance in the UV (d) corresponds to $R_{UVpeak.}$

Fig. 2: Path diagram showing the relative importance of different additive and interactive pathways potentially explaining variation in carotenoid-based plumage coloration. Shown are statistically significant treatment effects (outermost left) acting on three hierarchical levels: two levels of color components (left and middle) and one level of feather coloration (right), and their effects on plumage coloration (outermost right). By design, causal relationships exist between components of two different levels (unidirectional left to right arrows) and relationships between components of the same level are considered to be correlations (bidirectional arrows). Arrow width is proportional to the standardized partial correlation coefficients (β). For correlated variables, β can differ, depending on which one is hierarchically higher. Continuous lines depict positive and dashed lines negative effects. All effects that were statistically supported in ≥75 % of the path models are shown.

Fig. 3: Effects of the interaction between carotenoid supplementation (βLZ: β-carotene/lutein/zeaxantin; LZ: lutein/zeaxantin; C: control group) and immunization (I: Immunized; CI: control-injected group) on plumage hue (a) saturation (b) and brightness (c). Averages ±1 SE are shown. Significant least-square mean contrasts are depicted by asterisks (*$P < 0.05$; ** $P< 0.01$).

769 **Fig. 4:** Effects of experimental immunization on total feather length. Immunized
770 nestlings showed significantly shorter feathers.